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Author: Tatiana Andreani Charlene P. Kiill Ana Luiza R. de Souza Joana F. Fangueiro Lisete Fernandes Slavomira Doktorovová Dario L. Santos Maria L. Garcia Maria Palmira D. Gremião Eliana B. Souto Amélia M. Silva



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**Surface engineering of silica nanoparticles for oral insulin delivery:
characterization and cell toxicity studies**

**Tatiana Andreani^{a,b,c}, Charlene P. Kiill^d, Ana Luiza R. de Souza^d, Joana F.
Fangueiro^{c,e}, Lisete Fernandes^f, Slavomira Doktorovová^g, Dario L. Santos^{a,b}, Maria
L. Garcia^h, Maria Palmira D. Gremião^d, Eliana B. Soutoⁱ, Amélia M. Silva^{a,b,*}**

^aDepartment of Biology and Environment, University of Trás-os Montes e Alto Douro, UTAD, Quinta de Prados, 5001-801, Vila Real, Portugal

^bCentre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real, Portugal

^cFernando Pessoa University, UFP, Praça 9 de Abril, 349, 4249-004, Porto, Portugal

^dFaculty of Pharmaceutical Sciences, Universidade Estadual Paulista, UNESP, Rodovia Araraquara-Jau, Km. 01, Araraquara, São Paulo, Brazil

^eResearch Centre for Biomedicine, CEBIMED, Fernando Pessoa University, UFP, Porto, Portugal

^fElectron Microscopy Unit, UTAD, Vila Real, Portugal

^gResearch Institute For Medicines and Pharmaceutical Sciences – iMed.UL, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649-003, Lisbon, Portugal

^hDepartment of Physical Chemistry, Faculty of Pharmacy, Barcelona University, Av. Joan XXIII s/n, 08028 Barcelona, Spain

ⁱDepartament of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra (FFUC), Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

* Corresponding author:

Amélia M. Silva

Department of Biology and Environment, School of Life and Environmental Sciences, University of Trás-os-Montes and Alto Douro, Quinta de Prados; 5001-801 Vila Real; Portugal

E-mail: amsilva@utad.pt

Phone: +351 259350106; Fax: +351-259350480

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34

35 **Abstract**

36 The present work aimed at studying the interaction between insulin and SiNP surfaced
37 with mucoadhesive polymers (chitosan, sodium alginate or polyethylene glycol) and the
38 evaluation of their biocompatibility with HepG2 and Caco-2 cell lines, which mimic *in*
39 *vivo* the target of insulin-loaded nanoparticles upon oral administration. Thus, a
40 systematic physicochemical study of the surface-modified insulin-silica nanoparticles
41 (Ins-SiNP) using mucoadhesive polymers has been described. The surfacing of
42 nanoparticle involved the coating of silica nanoparticles (SiNP) with different
43 mucoadhesive polymers, to achieve high contact between the systems and the gut
44 mucosa to enhance the oral insulin bioavailability. SiNP were prepared by a modified
45 Stöber method at room temperature via hydrolysis and condensation of tetraethyl
46 orthosilicate (TEOS). Interaction between insulin and nanoparticles was assessed by
47 differential scanning calorimetry (DSC), X-ray and Fourier-transform infrared (FTIR)
48 studies. The high efficiency of nanoparticles' coating resulted in more stable system.
49 FTIR spectra of insulin-loaded nanoparticles showed amide absorption bands which are
50 characteristic of α -helix content. In general, all developed nanoparticles demonstrated
51 high biocompatible, at the tested concentrations (50 – 500 $\mu\text{g/mL}$), revealing no or low
52 toxicity in the two human cancer cell lines (HepG2 and Caco-2). In conclusion, the
53 developed insulin-loaded SiNP surfaced with mucoadhesive polymers demonstrated its
54 added value for oral administration of proteins.

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57 **Keywords:** silica nanoparticles, coated-SiNPs, insulin, mucoadhesive polymers, HepG2
58 cell, Caco-2 cell

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61 **1. Introduction**

62

63 The recent advances in the field of biotechnology has shown several innovative
64 strategies for protein drug delivery, exploiting non-invasive routes (e.g. oral [1, 2], nasal
65 [3], pulmonary [4, 5], buccal [6, 7] or transdermal [8]), to reach a better patient's
66 compliance in the treatment of diabetes. Among the proposed non-invasive alternatives,
67 the oral administration of insulin seems to be more convenient to the patient. Insulin
68 could be rapidly delivered in the liver through the portal circulation, after being
69 absorbed in the intestine and, thus, the hyperinsulinemia condition could be avoided [9].
70 Nevertheless, the oral absorption of therapeutic proteins is hindered by several
71 difficulties, such as their high molecular weight and hydrophilicity, low pH of gastric
72 medium leading to protein denaturation and the presence of proteolytic enzymes that
73 can reduce or even abolish their performance *in vivo* [10].

74 A promising strategy to improve the oral insulin bioavailability is to develop drug
75 delivery systems that protect the protein from metabolic degradation, as well as prolong
76 the gastrointestinal residence time, improving the absorption of the macromolecules
77 through the intestinal tract. Nanoparticles coated with selected mucoadhesive polymers
78 would be advantageous for oral delivery of therapeutic proteins.

79 Due to their high porosity, specific surface area, biocompatibility and ease of surface
80 functionalization, silica nanoparticles (SiNP) have been considered an excellent option
81 as delivery systems for proteins [11-14]. The presence of residual silanol groups (Si-OH)
82 onto the silica surface triggers the reactive sites for its surface modification by specific
83 organic groups [15].

84 Based on these previous considerations, the purpose of the present study was to develop
85 and characterize an organic/inorganic hybrid system intended for the oral insulin
86 administration by combining the advantages of SiNP with the mucoadhesive properties
87 of selected hydrophilic polymers. In this work, SiNP were chosen as drug delivery
88 system for insulin, using chitosan (CH), sodium alginate (SA) or poly(ethylene glycol)
89 (PEG) as mucoadhesive polymers. Multifunctional polymers have been extensively
90 explored as matrix material in the development of mucosal drug delivery systems [16].
91 Chitosan is a biocompatible polysaccharide which improves the penetration of
92 therapeutic proteins in the intestinal mucosa, because of the interaction of its amine

93 groups with negatively charged mucin. Chitosan can increase the paracellular
94 permeability by affecting the structure of proteins associated to the tight junctions [17].
95 Alginate is a biopolymer also showing bioadhesive properties. Unlike chitosan, alginate
96 prolongs the drug residence time in the mucosa due to the presence of numerous
97 carboxyl groups, leading to a strong bioadhesive interaction by hydrogen bonds
98 between anionic polymer and mucin [18].
99 PEG-coated nanoparticles have also been investigated for oral administration. It is
100 known that PEG coatings can stabilize the nanoparticles in the gastric and intestinal
101 fluids by steric hindrance, due to the inhibition of plasma protein adsorption [19]. In
102 addition, PEGs can promote the mucoadhesion by the penetration of their chains in the
103 intestinal mucosa [20].
104 Despite the increased attractiveness of nanotechnology for biomedical applications, the
105 human exposure and environmental impact of the nanomaterials are also of great
106 concern. Recent studies show that intrinsic properties of nanoparticles, such as size,
107 shape and surface charge, can damage the cell membrane leading to changes of cell
108 morphology and stability [21]. Therefore, it is important to consider a balance between
109 the benefits and the potential hazards of nanomaterials when developing a suitable
110 system for the purpose of drug and targeting delivery.
111 In the present study, the interaction between insulin and SiNP coated with different
112 mucoadhesive polymers was examined by X-ray diffraction, differential scanning
113 calorimetry (DSC) and Fourier transform infrared (FTIR) analyses. The
114 biocompatibility of different nanoparticles was evaluated in HepG2 and Caco-2 cell
115 lines, which mimic the *in vivo* the target of insulin-loaded SiNP upon oral
116 administration.
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119 **2. Materials and methods**

120

121 **2.1. Materials**

122

123 Tetraethyl orthosilicate (TEOS, 98%), NH₃ 25%, PEG with M_w of 6000 and 20000 Da
 124 (PEG 6000; PEG 20000) were purchased from Merck (Darmstadt, Germany). Chitosan
 125 low molecular weight (235 g/mol, deacetylation degree of 78.5 %), ethanol 99.9 %,
 126 trehalose dehydrate and bovine serum albumin (BSA) were purchased from Sigma-
 127 Aldrich (Steinheim, Germany). Sodium alginate (198.11 g/mol) was purchased from
 128 VWR Portugal (Carnaxide, Portugal). Solution of 100 IU/mL of human insulin
 129 (Humulin[®] R) was purchased from Eli Lilly (Lisbon, Portugal). Dulbecco's Modified
 130 Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin/streptomycin, L-
 131 glutamine, 0.05% trypsin-EDTA and AlamarBlue (AB) were purchased from Gibco
 132 (Alfagene, Invitrogen, Portugal). HepG2 (Human hepatocellular carcinoma cell line;
 133 ATCC[®] Number: HB-8065 TM) were a gift from Professor Carlos Palmeira (CNC-UC,
 134 Coimbra, Portugal) and Caco-2 (Human colon adenocarcinoma cell line) was purchased
 135 from Cell Lines Service (CLS, Eppelheim, Germany). Ultra-purified water was obtained
 136 from MiliQ Plus system (Milipore, Germany).

137

138 **2.2. Synthesis of nanoparticles**

139

140 Silica nanoparticles were synthesized at room temperature via hydrolysis and
 141 condensation of TEOS under high shear homogenization (Ultra-Turrax, IKA, T25)
 142 using NH₃ as catalytic agent. The obtained nanoparticles were centrifuged and washed
 143 with a mixture of ethanol and ultra-purified water (1:1, v/v) by 2 cycles at 12,000 rpm
 144 for 5 min (Spectrafuge16M, Lambnet International, Inc.).

145 For coating silica nanoparticles, a solution of chitosan (CH) (0.3%, w/v) at pH 4.5, or
 146 sodium alginate (SA) (0.3% , w/v) at pH 4.5, or PEG 60002 or PEG 20000 (2%, w/v) at
 147 pH 6.8 was added to the nanoparticles, stirred for 30 min and centrifuged as described
 148 above.

149 For insulin association to SiNP, 1 mL of human insulin (100 IU/mL, pH 7.0) was added
 150 to 10 mg of uncoated SiNP under gentle stirring (300 rpm) for 30 min into ice bath.

For coated insulin-SNP, 1.0 mL of human insulin (100 IU/mL, pH 7.0) was dissolved in 2 mL of the hydrophilic polymer solutions, mixed for 30 min under magnetic stirring and then added to SiNP (10 mg) under gentle stirring (300 rpm) for more 30 min into ice bath. The nanoparticles were centrifuged at 5000 rpm for 5 min and the pellet was freeze-dried during 24 h in the presence of trehalose (10 %, w/v).

2.3. Differential Scanning Calorimetry (DSC) analysis

Thermograms were obtained using a TA Instrument (New Castle, USA). Accurately, 5 mg of lyophilized nanoparticles were weighted in 40 μ L aluminium pans. DSC scans have been recorded from 25 to 350°C at a heating constant rate of 10°C/min under purging of nitrogen at 20 mL/min using an empty pan as reference. Data were obtained from the peaks areas using the TA software (TA Instrument).

2.4. Fourier Transform Infrared (FTIR) analysis

FTIR-spectra were performed using a Shimadzu® Europe - Prestige-21 spectrometer. Uncoated and coated nanoparticles containing insulin were gently mixed with a suitable amount of micronized KBr powder and compressed into discs at a force of 10 kN using a manual tablet presser. For each spectrum, a 128-scan interferogram was collected with a 4 cm^{-1} resolution in the mid-IR region at 25 °C.

2.5. X-Ray studies

X-Ray diffraction patterns of bulk materials and nanoparticles were performed using Siemens D5000 diffractometer system (Siemens, Germany) with a copper anode($\text{Cu-K}\alpha$ radiation, $\lambda = 0.1542 \text{ nm}$) at angles $2\theta = 4\text{-}70^\circ$.

2.6. Cell cultures and maintenance

HepG2, a human hepatocellular carcinoma cell line, obtained from ATCC, was kindly provided by Prof. Carlos Palmeira (CNC, UC, Portugal) and Caco-2, a human colorectal adenocarcinoma cell line, obtained from Cell Line Services, AG (Germany) were used as cell models to perform the cytotoxicity assay of the different nanoparticles. HepG2

185 and Caco-2 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium)
 186 supplemented with 10 % (v/v) foetal bovine serum (FBS), antibiotics (100 U/mL of
 187 penicillin and 100 µg/mL of streptomycin) and 1 mM L-glutamine in an atmosphere of
 188 5% CO₂/95 % air, at 37 °C with controlled humidity.

189

190 **2.7. *In vitro* cytotoxicity assay**

191

192 The cytotoxicity of nanoparticles was evaluated by comparing the proliferation rate and
 193 viability of non-exposed HepG2 or Caco-2 cells(control) with exposed HepG2 or Caco-
 194 2 cells, to appropriate concentrations during defined periods of time (see below), using
 195 the AB reduction method.

196 For the cytotoxicity assay cells were detached from the culture flasks with trypsin,
 197 counted and seeded into 96-well microplates at a density of 5×10⁴ cells/mL (100
 198 µL/well). Lyophilized nanoparticles were diluted in FBS-free culture media to various
 199 concentrations, ranging from 50 to 500 µg/mL (0, 50, 200, 500 µg/mL). Then, 24 h after
 200 seeding, the culture media was removed and replaced by media containing the
 201 nanoparticles (at defined concentrations). For each concentration of nanoparticles,
 202 microplates were placed in the incubator, and cells were exposed for 48 h. After the
 203 exposure time, the media containing the nanoparticles (and the control) was removed
 204 and replaced by FBS-free medium supplemented with 10 % (v/v) of AB. The
 205 absorbance readings occurred about 4 h after AB addition, at 570 and 620 nm using a
 206 Multiskan EX microplate reader (MTX Labsystems, USA). The percentage of AB
 207 reduction was calculated according to the following equation:

208

$$209 \quad \% \text{ AB reduction} = \frac{(\epsilon_{ox}\lambda_2)(A\lambda_2) - (\epsilon_{ox}\lambda_1)(A\lambda_1)}{(\epsilon_{red}\lambda_2)(A\lambda_2) - (\epsilon_{red}\lambda_1)(A\lambda_1)} \times 100$$

210

211 where, $\epsilon_{ox}\lambda_1$ is the molar extinction coefficient of oxidized AB at 570 nm, $\epsilon_{ox}\lambda_2$ is the
 212 molar extinction coefficient of oxidized AB at 620 nm, $\epsilon_{red}\lambda_1$ is the molar extinction
 213 coefficient of reduced AB at 570 nm, $\epsilon_{red}\lambda_2$ is the molar extinction coefficient of
 214 reduced AB at 620 nm, $A\lambda_1$ and $A\lambda_2$ are the absorbance of test wells at 570 and 620 nm,
 215 respectively, and $A\lambda_1$ and $A\lambda_2$ are the absorbance of the negative control wells (media
 216 plus AB but no cells) at 570 and 620 nm, respectively.

217

2.8. Statistical analysis

Results are expressed in terms of cell viability as percentage of control (untreated cells), and are a mean of three independent experiments ($n=3$) \pm S.D (in each experiment, each condition was tested in 8 replicate wells (octuplicates)). Statistically evaluation of data was performed using a one-way analysis of variance (ANOVA) test. Bonferroni's Multiple Comparison test was carried out to compare the significance between the different groups. A p -value < 0.05 was considered statistically significant.

3. Results and discussions

3.1. DSC analysis

In this study, DSC was used to evaluate the influence of the selected coatings in SiNP. Table 1 presents a summary of the peak temperature and enthalpies associated with each peak for the various bulk materials and for the nanoparticles produced by sol-gel technology.

Typical DSC thermograms of coated SiNP with sodium alginate, SiNP-SA, chitosan, SiNP-CH, PEG 6000, SiNP-PEG 6000, and PEG 20000, SiNP-PEG 20000, are shown in Figure 1A (as denoted). Thermogram of sodium alginate depicted an endothermic peak at 97 °C followed by an exothermic transition at 239.74 °C (Table 1). The exothermic peaks attributed to a polymer were attributed to the degradation phenomena due to depolymerization or oxidation reactions [22, 23].

[Please, insert Table 1 near here]

Upon coating of SiNP with SA (SiNP-SA), the endothermic peak was shifted to 139.91 °C associated to an enthalpy of 65.41 J/g (Figure 1A, Table 1). Also, the addition of SA onto SiNP surface shifted the exothermic peak to higher temperatures, in comparison to SA alone. The shift of melting point and the exothermic peak in SiNP-SA may ascribe to the interaction between silica and sodium alginate resulting in higher thermal stability of the system. The second endothermic peak, at 253.51 °C, was attributed to the removal of the absorbed water in the sample under heating.

252

253 **[Please, insert Figure 1 near here]**

254

255 From the DSC results, Ins-SiNP-SA (Figure 1B and Table 1) showed the endothermic
 256 peak at different temperature values comparing to that obtained with unloaded
 257 nanoparticles (Figure 1A). The endothermic peak started at lower temperature,
 258 confirming that the presence of insulin changes the thermal behavior of nanoparticles
 259 due to the interaction between the protein and the polymer.

260 As indicated on Table 1, chitosan exhibits a sharp endothermic event, ascribing to the
 261 melting peak around 88.94 °C and an exothermic event at 304.03 °C. The coating of
 262 SiNP with chitosan (Figure 1A) changed the thermal behavior of the polymer with
 263 respect to the bulk material, shifting the endothermic transition to higher temperatures
 264 (93.05 °C), indicating the formation of strong hydrogen bonding between silica and
 265 chitosan [24]. To note, the exothermic peak of chitosan disappeared. Upon insulin
 266 association to SiNP-CH (Figure 1B), the endothermic peak is still present, almost
 267 unmodified. It can be concluded that the coating of SiNP with chitosan resulted in
 268 higher stability of the system, requiring more energy to break the interactions between
 269 silica and the polymer, as well as during the thermal decomposition of the nanoparticles.
 270 Again, the second endothermic peak at 204.98 °C observed after coating with chitosan
 271 was due to the removal of adsorbed water. No signal of insulin peak was detectable after
 272 its incorporation in SiNP-CH, suggesting that insulin is completely dissolved in the
 273 polymer chains leading to an interaction between insulin and the polyelectrolyte (Figure
 274 1; Table 1).

275 Concerning the effect of PEGylation on SiNP, the thermal behavior of nanoparticles
 276 using PEG 6000 was similar to that using PEG 20000 (Figure 1A). Pure PEG 6000 and
 277 PEG 20000 melt at 63.04 and 54.43 °C, respectively (Table 1). The coating of SiNP
 278 with PEG 6000 and PEG 20000 shifted the endothermic peaks of the polymers to higher
 279 temperatures about 83.41 °C and 94.84 °C, respectively (Figure 1A). This result was
 280 attributed to the fact that PEG chains, in PEG-SiNP, are less flexible than those in pure
 281 PEG due to the interaction between silica and PEG segments (Figure 1A, Table 1).
 282 SiNP could act as nucleating agent, promoting the orientation of PEG chains and
 283 consequently leading to the high formation of crystal. In the presence of insulin, the
 284 endothermic peaks were registered at 92.10 °C for Ins-SiNP-PEG 6000 (Figure 1B) and
 285 at 102.17 °C for Ins-SiNP-PEG 20000 (Figure 1B). However, the peak recorded around

50 °C, observed in both formulations, can be related to the transition midpoint (T_m) of insulin. T_m of insulin in the absence of nanoparticles was found to be 77.64 °C (data not shown). Therefore, it is clear that PEG decreased the thermal stability of insulin. These results are in agreement with other studies that indicate that PEGs interact with the protein molecules by hydrophobic interactions being responsible for the destabilization of the protein structure [25].

3.2. X-ray studies

X-ray diffraction spectra indicate that sodium alginate, chitosan, PEG 6000, and PEG 20000 are present as a crystalline material (data not shown). However, the intensity of the peaks in SiNP-coated with the polymers is decreased, reflecting less ordered structure of the nanoparticles (Figures 2A). The association of insulin to nanoparticles also supports high crystallinity of the nanoparticles in comparison to unloaded nanoparticles (Figures 2B). The solubilization of insulin into the polymer solutions may have a tendency to crystallize the formulations during storage, thus leading to a change in the physical properties of the nanoparticles.

[Please, insert Figure 2 near here]

3.3. FTIR analysis

Figure 3 shows the FTIR spectra relative to the Ins-SiNP-CH (a), Ins-SiNP-SA (b), Ins-SiNP-PEG 6000 (c), Ins-SiNP-PEG 20000 (d) and Ins-SiNP (e).

The FTIR spectra of Ins-SiNP (Figure 3 (e)) showed a peak of free O–H stretching vibration around 3500 cm^{-1} (H-bonded H_2O , hydroxyl terminals, H-bonded OH vibrations of alcohol and H-bonded Si–OH in chain), a peak of Si–O stretching vibration around 1040 cm^{-1} , a peak Si–OH at 980 cm^{-1} , and a peak of Si–O–Si bending around 600 cm^{-1} vibration [26, 27].

The spectra of Ins-SiNP-CH (Figure 3 (a)) showed the presence of peaks around 1600, 1500 and 1400 cm^{-1} , related to amide bond, to vibration of protonated amine group and $-\text{CH}_2$ bending, respectively. The absorption bands at 1000 cm^{-1} (skeletal vibrations involving the C–O stretching) are characteristics of its saccharide structure [28]. A

characteristic band at 3440 cm^{-1} was assigned to O–H stretching, indicating intermolecular hydrogen bonding which is overlapped in the same region to the stretching vibration of N–H.

The bands around 1600 and 1400 cm^{-1} present in the FTIR spectrum of Ins-SiNP-SA Figure 3 (b) are assigned to symmetric and asymmetric stretching vibrations of carboxylate salt groups. In addition, the bands around 1300 cm^{-1} (C–O stretching), 1100 cm^{-1} (C–C stretching) and 1000 cm^{-1} (C–O stretching) are attributed to its saccharide structure [29].

The representative FTIR spectra of Ins-SiNP-PEG 20000 (Figure 3 (c)) and Ins-SiNP-PEG 6000 (Figure 3 (d)) were quite similar. The region between 3300 and 3600 cm^{-1} corresponds to O–H stretching, the band ranging from 2800 to 2900 cm^{-1} corresponds to C–H stretching and the band between 1000 and 1200 cm^{-1} is assigned to C–O stretching. The addition of PEG increased the relative intensity of the OH band indicating the increase of degree of hydration of the samples [30].

333

334 **[Please, insert Figure 3 near here]**

335

Comparing the spectra, changes observed in the absorption band of O–H can be assumed as a possible interaction that would occur between OH groups of SiNP and OH groups of PEG, as well as between OH groups and carboxyl of alginate or amino groups of chitosan. These results suggest an effective interaction between silica and the polymers. Finally, a band indicative of amide I at 1645 cm^{-1} (C=O stretching) was observed in all samples, which is characterized by the presence of α -helical content [31].

343

344 **3.4. *In vitro* cytotoxicity assay**

345

In this study, the toxicity of the different nanoparticles was evaluated by the resazurin (Alamar Blue, AB) reduction assay using HepG2 and Caco-2 cell lines and results of cell viability are compared with those of non-exposed cells (control) in terms of % of control. Most authors consider that viability above 70% of the control is an indication of “no toxicity” or of a safe material, and only viability below 70% is considered toxic, as reviewed recently [32].

351

Caco-2 and HepG2 were exposed for 48 h to SiNP, to coated-SiNP and to insulin loaded coated-SiNP, and the obtained results are shown in the Figures 4, 5 and 6. For the uncoated SiNP, cell viability ranged from 97.67 ± 0.19 % (for 50 $\mu\text{g/mL}$) to 108.97 ± 2.17 % (for 200 $\mu\text{g/mL}$) for Caco-2 cells (Figure 4A), and from 92.63 ± 1.04 % (for 50 $\mu\text{g/mL}$) to 101.45 ± 3.41 % (for 200 $\mu\text{g/mL}$) for HepG2 cells (Figure 4B). No statistical significant changes, compared with the control group, were observed in both cell lines and at all tested concentrations, after 48 h exposure to uncoated SiNP ($p > 0.05$). These results can be attributed to the surface charge of nanoparticles. In this case, SiNP have negative charge at pH 7. Several studies have reported that negatively charged nanoparticles exert very little or no toxicity on biological membranes, in comparison to positively charged particles [33]. In Figure 4, a slight decrease in cell viability is observed when cells are exposed to insulin-loaded nanoparticles, compared to control. The decrease is more evident in Caco-2 cells (Figure 4A) than in HepG2 cells (Figure 4B), but the differences are minimal (no more than a 10% of decrease, from control).

[Please, insert Figure 4 near here]

It is important to consider that the coating of the nanoparticles with chitosan (CH), sodium alginate (SA) or PEG may change the pattern of toxicity comparing with the free polymers. For SiNP coated with chitosan, the cell viability ranged from 76.68 ± 1.17 % (for 500 $\mu\text{g/mL}$) to 96.84 ± 0.97 % (for 50 $\mu\text{g/mL}$) for Caco-2 cell (Figure 5A) and from 85.99 ± 8.99 % (for 200 $\mu\text{g/mL}$) to 99.44 ± 2.91 % (for 50 $\mu\text{g/mL}$) for HepG2 cells (Figure 5B). As shown in Figure 5A, compared with the control group, all concentrations reduced significantly the cell viability ($p < 0.05$), although reduction in not higher than 25 %. Figure 5A also shows that cell viability is reduced with the increase in the nanoparticles concentration, leading to cytotoxicity being concentration dependent, and that loading SiNP-CH with insulin improves cell viability.

As observed in Figure 5B, HepG2 cell viability was less affected by nanoparticles exposure than Caco-2 cells. At 50 $\mu\text{g/mL}$ SiNP-CH, changes in cell viability were not statistically significant, after 48 h exposure. Comparing with uncoated SiNP (Figure 4), it is observed that SiNP coated with chitosan (SiNP-CH) induced slightly higher toxicity in both cell lines (Figure 5A and B). *In vitro* evaluation of chitosan and chitosan nanoparticles has been performed in a wide range of cell lines demonstrating low

386 cytotoxicity [34, 35]. However, it is known that cationic compounds can cause cell
 387 damage. The presence of the positive charges on the SiNP-CH surface may
 388 consequently affects the interaction with cells leading to a decrease of cell viability.
 389 Many studies suggest that cationic materials imply higher toxicity due to the
 390 interactions with the plasmatic membrane and/or with negatively charged cell
 391 components and proteins [36-38]. Also, some works have showed that chitosan coated
 392 nanoparticles can induce cell apoptosis in some extend [39].

393 As observed in Figure 5A and 5B, insulin-loaded nanoparticles decreased the
 394 cytotoxicity of SiNP-CH, after 48 h of exposure, being more evident in Caco-2 cells at
 395 high concentrations. This phenomenon can be related to the possible decrease of the
 396 interaction between the positively charged amino groups of chitosan with the anionic
 397 components of the glycoproteins on the cell membrane surface, improving cell viability.
 398 Regarding to SiNP-SA, Caco-2 cells (Figure 5C) were also more susceptible than
 399 HepG2 cells (Figure 5D) to the exposure to SiNP-coated with sodium alginate. All
 400 concentrations of SiNP-SA significantly reduced the viability of Caco-2 cells, compared
 401 to control (Figure 5C), however, some reductions are minimal (no more than a 20% of
 402 decrease, from control). However, only the concentration of 500 µg/mL of SiNP-SA
 403 reduced significantly HepG2 viability (Figure 5D). Similar results were obtained by
 404 Douglas and co-workers [40], demonstrating that high concentrations of alginate-
 405 chitosan nanoparticles resulted in a significant decrease of 293 T cells viability after 24
 406 h of incubation in comparison to chitosan polymer.

407

408 **[Please, insert Figure 5 near here]**

409

410 After insulin incorporation into SiNP-SA, all tested concentrations showed low
 411 cytotoxicity in Caco-2 (Figure 5C) and HepG2 cells (Figure 5D). As reported
 412 previously, insulin loading seems to improve cell viability. These results are in evident
 413 agreement with other studies demonstrating high biocompatibility of alginate as a
 414 coating or even as a carrier [41].

415 In general, we can observe a low degree of toxicity for all particles at the concentration
 416 range tested. However, for the unloaded nanoparticles, a reduction in the cell viability is
 417 observed which is concentration dependent, and it is more evident for Caco-2 cells, as
 418 observed for SiNP-CH (Figure 5A) and for SiNP-SA (Figure 5C).

Concerning the coating with PEG (Figure 6), two different PEG polymers were studied differing in chain extent and thus in MW, the PEG 6000 and PEG 20000 were used. Cell viability of Caco-2 (Figure 6A) and HepG2 cells (Figure 6B) after 48 h of exposure to SiNP-PEG 6000 and SiNP-PEG 20000 is shown. Concerning the effect of nanoparticles concentration on cytotoxicity, we could note that higher concentrations of SiNP-PEG 6000 (200 and 500 $\mu\text{g/mL}$) induced higher cytotoxicity in both cell lines (Figure 6A and 6B), to note that 500 $\mu\text{g/mL}$ of SiNP-PEG 6000 reduced Caco-2 cell viability by more than 50% (Figure 6A). However, according to what has been already reported insulin-loading improves cell viability, and for all concentrations of Ins-SiNP-PEG 6000 viability is around 90%.

On the other hand, SiNP-PEG 20000 did not significantly affect the HepG2 or the Caco-2 cell viability (Figure 6B and 6A). In general, conjugation of PEG to nanoparticles is recognized as being nontoxic by all routes of administration. However, comparing to uncoated SiNP, the concentrations of 200 and 500 $\mu\text{g/mL}$ of SiNP-PEG 6000, in both cell lines, reduced significantly cell viability. Cho and co-workers [42] showed that gold nanoparticles coated with PEG 5000 can induce acute inflammation and apoptosis in the mouse liver. Higher cell viability, especially at concentration of 200 and 500 $\mu\text{g/mL}$ was observed for SiNP-PEG 20000 compared to that of SiNP-PEG 6000. This result can be attributed to the long chain structure of PEG 20000 leading to a higher steric effect. The flexibility of a long PEG chain like PEG 20000 was supposed to make it to cover greater surface area. Similar observations were obtained by Mao and co-workers [43] that verified lower cytotoxicity effect of trimethyl chitosan (TMC) grafted by PEG 5 kDa in comparison to TMC grafted by PEG 550 Da. However, some studies showed that low PEG chain length used for poly(ethylene imine) (PEI) coating induced low cytotoxic and oxidative stress response in lung cell line [44]. No cytotoxicity effect on cell proliferation and viability was observed after the incorporation of insulin into SiNP-PEG 6000 and SiNP-PEG 20000 for both cell lines (Figure 6).

446

447 **4. Conclusions**

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DSC, X-ray and FTIR were used to evaluate the influence of different coatings in insulin-loaded SiNP. In the DSC studies, the endothermic and exothermic peaks of pure polymers were shifted to high temperature in all coated SiNP resulting in more stable systems. The X-ray diffraction showed that coated SiNP displayed less ordered

structure compared with pure polymers. On the other hand, the association of insulin to nanoparticles resulted in more crystalline structures. FTIR analysis also demonstrated the interaction between mucoadhesive polymers and nanoparticles. Additionally, the toxicity assay showed that different surface modification of SiNP did not affect, or affect in a low degree, the cell viability, demonstrating very low toxicity in Caco-2 and HepG2 cell lines, indicating that the developed nanoparticles are promising biocompatible for oral drug delivery systems.

Acknowledgments

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References

- [1] F.-Y. Su, K.-J. Lin, K. Sonaje, S.-P. Wey, T.-C. Yen, Y.-C. Ho, N. Panda, E.-Y. Chuang, B. Maiti, H.-W. Sung, Protease inhibition and absorption enhancement by functional nanoparticles for effective oral insulin delivery, *Biomaterials*, 33 (2012) 2801-2811.
- [2] N. Shrestha, M.-A. Shahbazi, F. Araújo, H. Zhang, E.M. Mäkilä, J. Kauppila, B. Sarmiento, J.J. Salonen, J.T. Hirvonen, H.A. Santos, Chitosan-modified porous silicon microparticles for enhanced permeability of insulin across intestinal cell monolayers, *Biomaterials*, 35 (2014) 7172-7179.
- [3] E.-S. Khafagy, N. Kamei, E.J.B. Nielsen, R. Nishio, M. Takeda-Morishita, One-month subchronic toxicity study of cell-penetrating peptides for insulin nasal delivery in rats, *Eur J Pharm Biopharm*, 85(2013) 736-743.
- [4] S. Al-Qadi, A. Grenha, D. Carrión-Recio, B. Seijo, C. Remuñán-López, Microencapsulated chitosan nanoparticles for pulmonary protein delivery: In vivo evaluation of insulin-loaded formulations, *J Control Release*, 157 (2012) 383-390.

- 487 [5] K.P. Amancha, S. Balkundi, Y. Lvov, A. Hussain, Pulmonary sustained release of
 488 insulin from microparticles composed of polyelectrolyte layer-by-layer assembly, *Int*
 489 *J Pharm*, 466 (2014) 96-108.
- 490 [6] C. Giovino, I. Ayensu, J. Tetteh, J.S. Boateng, Development and characterisation of
 491 chitosan films impregnated with insulin loaded PEG-b-PLA nanoparticles (NPs): A
 492 potential approach for buccal delivery of macromolecules, *Int J Pharm*, 428 (2012)
 493 143-151.
- 494 [7] J.O. Morales, S. Huang, R.O. Williams, J.T. McConville, Films loaded with Insulin-
 495 coated nanoparticles (ICNP) as potential platforms for peptide buccal delivery,
 496 *Colloids Surf B Biointerfaces*, 122 (2014) 38-45.
- 497 [8] S. Liu, M.-n. Jin, Y.-s. Quan, F. Kamiyama, H. Katsumi, T. Sakane, A. Yamamoto,
 498 The development and characteristics of novel microneedle arrays fabricated from
 499 hyaluronic acid, and their application in the transdermal delivery of insulin, *J Control*
 500 *Release*, 161 (2012) 933-941.
- 501 [9] D.R. Owens, B. Zinman, G. Bolli, Alternative routes of insulin delivery, *Diabet*
 502 *Med*, 20 (2003) 886-898.
- 503 [10] J. Hamman, G. Enslin, A. Kotzé, Oral delivery of peptide drugs, *BioDrugs*, 19
 504 (2005) 165-177.
- 505 [11] Z. Deng, Z. Zhen, X. Hu, S. Wu, Z. Xu, P.K. Chu, Hollow chitosan-silica
 506 nanospheres as pH-sensitive targeted delivery carriers in breast cancer therapy,
 507 *Biomaterials*, 32 (2011) 4976-4986.
- 508 [12] D. Liu, X. He, K. Wang, C. He, H. Shi, L. Jian, Biocompatible silica nanoparticles-
 509 insulin conjugates for mesenchymal stem cell adipogenic differentiation, *Bioconjug*
 510 *Chem*, 21 (2010) 1673-1684.
- 511 [13] R.N. Jain, X. Huang, S. Das, R. Silva, V. Ivanova, T. Minko, T. Asefa,
 512 Functionalized mesoporous silica nanoparticles for glucose- and pH-stimulated
 513 release of insulin, *Z Anorg Allg Chem*, 640 (2014) 616-623.
- 514 [14] T. Andreani, A.L.R. Souza, C.P. Kiill, E.N. Lorenzón, J.F. Fangueiro, A.C.
 515 Calpena, M.V. Chaud, M.L. Garcia, M.P.D. Gremião, A.M. Silva, E.B. Souto,
 516 Preparation and characterization of PEG-coatedsilicananoparticlesfor oral
 517 insulin delivery, *Int J Pharm*, 473 (2014) 627-635.
- 518 [15] S.L. Westcott, S.J. Oldenburg, T.R. Lee, N.J. Halas, Formation and adsorption of
 519 clusters of gold nanoparticles onto functionalized silica nanoparticle surfaces,
 520 *Langmuir*, 14 (1998) 5396-5401.
- 521 [16] L. Yin, J. Ding, C. He, L. Cui, C. Tang, C. Yin, Drug permeability and
 522 mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin
 523 delivery, *Biomaterials*, 30 (2009) 5691-5700.
- 524 [17] Schipper NG, Olsson S, Hoogstraate JA, deBoer AG, Varum KM, A. P., Chitosans
 525 as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption
 526 enhancement, *Pharm Res*, 14 (1997) 923-929.

- 527 [18] K. Park, J.R. Robinson, Bioadhesive polymers as platforms for oral-controlled
528 drug delivery: Method to study bioadhesion, *Int J Pharm*, 19 (1984) 107-127.
- 529 [19] M. Tobío, A. Sánchez, A. Vila, I. Soriano, C. Evora, J.L. Vila-Jato, M.J. Alonso,
530 The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA
531 nanoparticles following oral administration, *Colloids Surf B*, 18 (2000) 315-323.
- 532 [20] N.A. Peppas, Molecular calculations of poly(ethyleneglycol) transport across a
533 swollen poly(acrylicacid)/mucin interface, *J Biomater SciPolymer Edn*, 9 (1998)
534 535-542.
- 535 [21] V.V. Ginzburg, S. Balijepalli, Modeling the thermodynamics of the interaction of
536 nanoparticles with cell membranes, *Nano Lett*, 7 (2007) 3716-3722.
- 537 [22] T. Mimmo, C. Marzadori, D. Montecchio, C. Gessa, Characterisation of Ca- and
538 Al-pectate gels by thermal analysis and FTIR spectroscopy, *Carbohydr Res*, 340
539 (2005) 2510-2519.
- 540 [23] M.J. Zohuriaan, F. Shokrolahi, Thermal studies on natural and modified gums,
541 *Polymer Testing*, 23 (2004) 575-579.
- 542 [24] S.M. Lai, A.J.M. Yang, W.C. Chen, J.F. Hsiao, The Properties and Preparation of
543 Chitosan/Silica Hybrids Using Sol-Gel Process, *Polym Plast Technol Eng*, 45 (2006)
544 997-1003.
- 545 [25] T. Arakawa, S.N. Timasheff, Mechanism of poly(ethyleneglycol) interaction of
546 proteins, *Biochemistry* 24 (1985) 6756-6762.
- 547 [26] K. Kusakabe, K. Ichiki, J.-I. Hayashi, H. Maeda, S. Morooka, Preparation and
548 characterization of silica-polyimide composite membranes coated on porous tubes
549 for CO₂ separation, *J Membr Sci*, 115 (1996) 65-75.
- 550 [27] A. Kioul, L. Mascia, Compatibility of polyimide-silicate ceramers induced by
551 alkoxysilane coupling agents, *J Non-Cryst Solids*, 175 (1994) 169-186.
- 552 [28] Y.J. Yin, K.D. Yao, G.X. Cheng, J.B. Ma, Properties of polyelectrolyte complex
553 films of chitosan and gelatin, *Polym Int*, 48 (1999) 429-432.
- 554 [29] C. Sartori, D.S. Finch, B. Ralph, K. Gilding, Determination of the cation content of
555 alginate thin films by FTIR spectroscopy, *Polymer*, 38 (1997) 43-51.
- 556 [30] M. Prokopowicz, J. Łukasiak, Synthesis and in vitro characterization of freeze-
557 dried doxorubicin-loaded silica/PEG composite, *J Non-Cryst Solids*, 356 (2010)
558 1711-1720.
- 559 [31] X.G. Zhang, D.Y. Teng, Z.M. Wu, X. Wang, Z. Wang, D.M. Yu, C.X. Li, PEG-
560 grafted chitosan nanoparticles as a injectable carrier for sustained protein release, *J
561 Mater Sci Mater Med*, 19 (2008) 3525-3533.
- 562 [32] S. Doktorovova, E.B. Souto, A.M. Silva, Nanotoxicology applied to solid lipid
563 nanoparticles and nanostructured lipid carriers – A systematic review of in vitro data,
564 *Eur J Pharm Biopharm*, 87 (2014) 1-18.

- [33] S. Bhattacharjee, L. de Haan, N. Evers, X. Jiang, A. Marcelis, H. Zuilhof, I. Rietjens, G. Alink, Role of surface charge and oxidative stress in cytotoxicity of organic monolayer-coated silicon nanoparticles towards macrophage NR8383 cells, *Particle Fibre Toxicol*, 7 (2010) 25.
- [34] M. Huang, E. Khor, L.-Y. Lim, Uptake and Cytotoxicity of Chitosan Molecules and Nanoparticles: Effects of Molecular Weight and Degree of Deacetylation, *Pharm Res*, 21 (2004) 344-353.
- [35] D. Jeevitha, K. Amarnath, Chitosan/PLA nanoparticles as a novel carrier for the delivery of anthraquinone: Synthesis, characterization and in vitro cytotoxicity evaluation, *Colloids Surf B*, 101 (2014) 126-134.
- [36] S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, M. Hashida, In vitro cytotoxicity of macromolecules in different cell culture systems, *J Control Release*, 34 (1995) 233-241.
- [37] D. Fischer, Y. Li, B. Ahlemeyer, J. Kriegelstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, *Biomaterials*, 24 (2003) 1121-1131.
- [38] J.F. Fanguiero, T. Andreani, M.A. Egea, M.L. Garcia, S.B. Souto, A.M. Silva, E.B. Souto, Design of cationic lipid nanoparticles for ocular delivery: Development, characterization and cytotoxicity, *Int J Pharm*, 461 (2014) 64-73.
- [39] L. Zhu, J. Ma, N. Jia, Y. Zhao, H. Shen, Chitosan-coated magnetic nanoparticles as carriers of 5-Fluorouracil: Preparation, characterization and cytotoxicity studies, *Colloids Surf B*, 68 (2009) 1-6.
- [40] K.L. Douglas, C.A. Piccirillo, M. Tabrizian, Effects of alginate inclusion on the vector properties of chitosan-based nanoparticles, *J Control Release*, 115 (2006) 354-361.
- [41] O. Borges, A. Cordeiro-da-Silva, S.G. Romeijn, M. Amidi, A. de Sousa, G. Borchard, H.E. Junginger, Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination, *J Control Release*, 114 (2006) 348-358.
- [42] W.-S. Cho, M. Cho, J. Jeong, M. Choi, H.-Y. Cho, B.S. Han, S.H. Kim, H.O. Kim, Y.T. Lim, B.H. Chung, J. Jeong, Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles, *Toxicol Appl Pharmacol*, 236 (2009) 16-24.
- [43] S. Mao, X. Shuai, F. Unger, M. Wittmar, X. Xie, T. Kissel, Synthesis, characterization and cytotoxicity of poly(ethyleneglycol)-graft-trimethylchitosan block copolymers, *Biomaterials* 26 (2005) 6343-6356.
- [44] A. Beyerle, O. Merkel, T. Stoecker, T. Kissel, PEGylation affects cytotoxicity and cell-compatibility of poly(ethyleneimine) for lung application: Structure–function relationships, *Toxicol Appl Pharmacol*, 242 (2010) 146-154.

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Figure Caption

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Figure 1. DSC thermograms. (A) SiNP coated with sodium alginate (SA), chitosan (CH), PEG 6000 or PEG 20000, as indicated. (B) SiNP coated with sodium alginate (SA), chitosan (CH), PEG 6000 or PEG 20000 after insulin association, as indicated.

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Figure 2. X-ray diffraction patterns of (A) SiNP coated with sodium alginate (SA), chitosan (CH), PEG 6000 or PEG 20000; (B) of SiNP coated with sodium alginate (SA), chitosan (CH), PEG 6000 or PEG 20000 after insulin association, as indicated.

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Figure 3. FTIR spectra of (a) Ins-SiNP-CH, (b) Ins-SiNP-SA, (c) Ins-SiNP-PEG 20000, (d) Ins-SiNP-PEG 6000 and (e) Ins-SiNP.

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Figure 4. Viability of Caco-2 (A) and HepG2 (B) cells after 48 h exposure to 50, 100, 200 and 500 µg/mL of uncoated and unloaded SiNP, (white bars) and uncoated insulin-loaded (grey bars). Cell viability is expressed as % of control, being the mean of 3 different experiments ± S.D.. For each cell line, three independent experiments (each with 8 replicates) were carried out.

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Figure 5. Effect of coating SiNP with chitosan (CH) or sodium alginate (SA) on cell viability. Caco-2 cells (A) and HepG2 cells (B) were exposed to empty chitosan coated SiNP (SiNP-CH; white bars) and to insulin loaded SiNP-CH (Ins-SiNP-CH; dark-grey bars). In different experiments, Caco-2 cells (C) and HepG2 cells (D) were exposed to SA-coated empty SiNP (SiNP-SA; light grey bars) or to insulin-loaded SiNP-SA (Ins-SiNP-SA; black bars). All cells were exposed to the respective NP for 48 h at 50, 100,

628

629 200 and 500 $\mu\text{g/mL}$, as denoted. Cell viability is expressed as % of control, being the
630 mean of 3 different experiments \pm S.D. For each cell line, three independent
631 experiments (each with 8 replicates) were carried out.

632

633 **Figure 6.** Effect of PEGylation of SiNP on the cell viability of Caco-2 (A) and HepG2
634 (B) cells using PEG 6000 and PEG 20000, as indicated. Cells were exposed for 48 h to
635 50, 100, 200 and 500 $\mu\text{g/mL}$ with coated-SiNP and insulin-loaded coated-SiNP, as
636 denoted. Cell viability is expressed as % of control being the mean of 3 different
637 experiments \pm S.D. For each cell line, three independent experiments (each with 8
638 replicates) were carried out.

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Table Caption

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644 **Table 1.** DSC parameters of the polymers and unloaded and loaded-nanoparticles
645 produced by sol-gel technology.

646

Table Caption

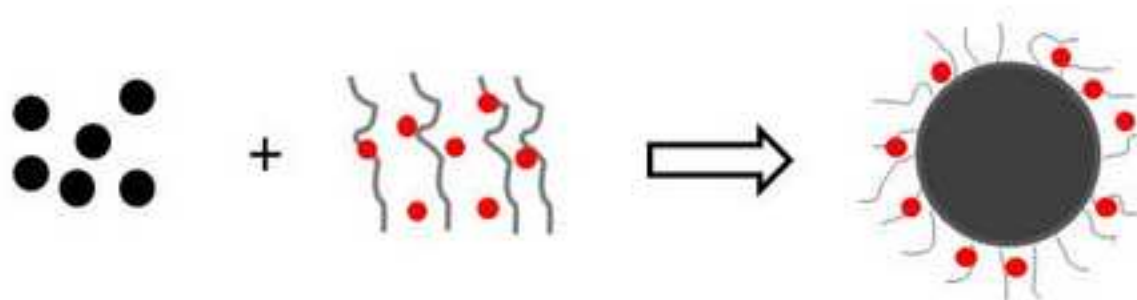
Table 1. DSC parameters of the polymers and unloaded and loaded-nanoparticles produced by sol-gel technology.

Table 1.

Samples	Temperature (°C)			ΔH (J/g)
	Initial	Peak	Final	
PEG 6000	58.50	63.04	73.57	163.40
PEG 20000	51.97	54.43	62.95	101.70
Sodium alginate	43.99	97.00	153.31	238.00
	220.78	239.74	273.67	202.70
Chitosan	45.17	88.94	139.72	124.40
	286.32	304.03	338.78	117.00
SiNP-PEG 6000	32.05	83.41	133.08	89.24
	268.72	287.30	347.28	145.40
SiNP-PEG 20000	40.84	94.84	159.43	123.40
	263.37	287.89	347.07	209.60
SiNP-SA	77.65	139.91	199.85	65.41
	210.11	253.51	279.06	26.69
	282.50	311.55	345.00	120.10
SiNP-CH	40.09	93.05	155.48	94.81
	201.29	229.84	306.84	126.20
Ins-SiNP-PEG 6000	59.63	92.10	114.01	128.70
Ins-SiNP-PEG 20000	69.28	101.17	142.21	146.10
Ins-SiNP-SA	78.23	102.16	129.77	132.30
	287.90	292.99	297.72	53.43
Ins-SiNP-CH	53.41	91.29	150.92	234.80
	193.02	204.98	230.12	133.30

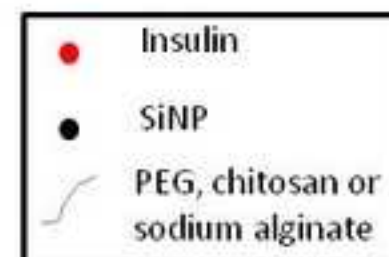
- 653 1) Hybrid nanoparticles were produced by Stöber method for insulin association
- 654 2) Silica nanoparticles were coated with chitosan, sodium alginate or PEG
- 655 3) Coating of silica nanoparticles resulted in more stable systems
- 656 4) PEGylated nanoparticles decreased the thermal stability of insulin
- 657 5) In general, all nanoparticles showed low toxicity in Caco-2 and HepG-2 cells
- 658

1) Production of coated-SiNP for insulin association



2) Characterization of nanoparticles

- Thermal analysis
- Crystallinity
- FTIR analysis



3) Biocompatibility of nanoparticles

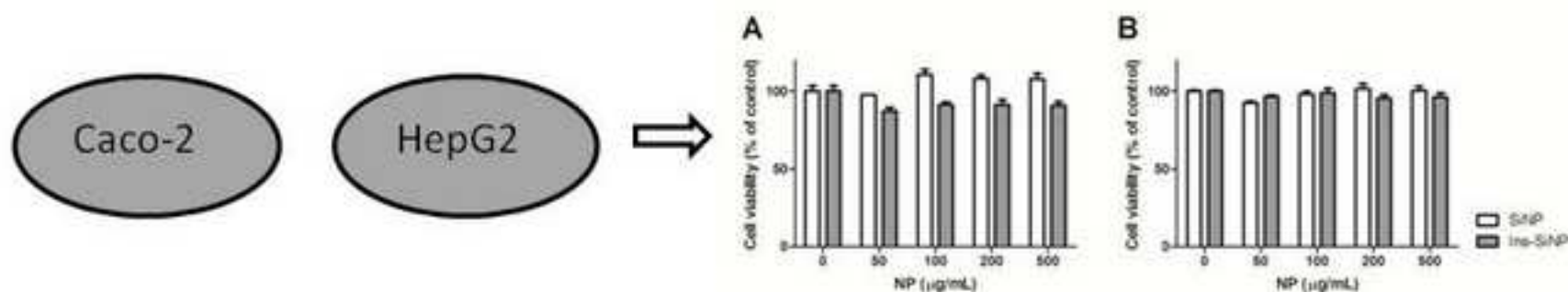


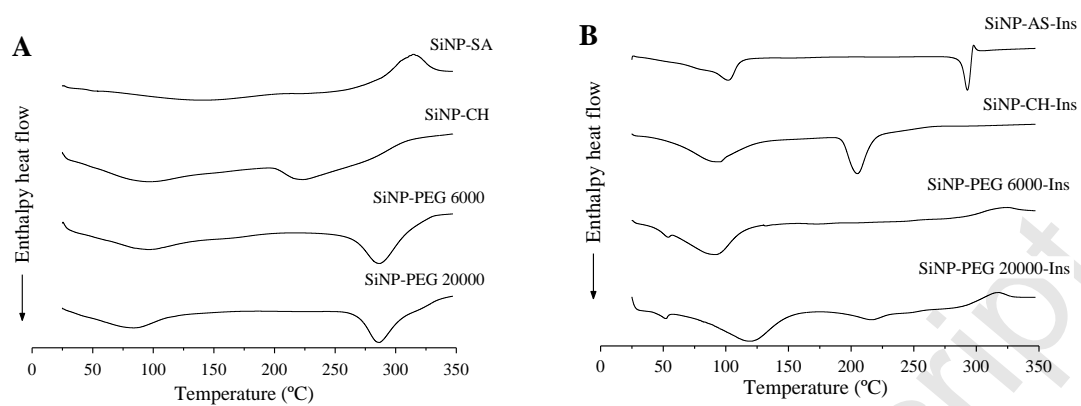
Figure 1.

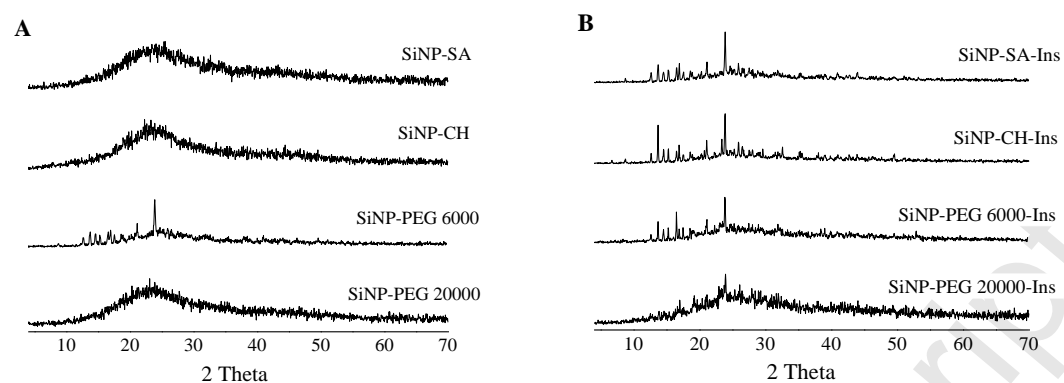
Figure 2.

Figure 3.